

# Tumor antigen analysis in neuroblastoma by serological interrogation of bioinformatic data

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The identification of tumor antigens remains a major objective in tumor immunology, especially in pediatric malignancies where solid tumors often do not express a single dominant antigen. Methods such as the Serological Screening of Recombinant cDNA Expression Libraries (SEREX) have been used in the discovery of tumor-expressed proteins by virtue of their ability to induce an antibody response. To focus and accelerate this approach, we first identified candidate antigens by gene expression profiling data from clinical neuroblastoma specimens and then used an animal model to generate an antibody response to an engineered cell-based vaccine. Candidate tumor antigens were expressed as recombinant proteins in a mammalian system and screened for antibody recognition using serum from mice vaccinated with a neuroblastoma cell-based vaccine engineered to express CD80 and CD86, with or without Treg depletion. Through this procedure, the never in mitosis A (NIMA)-related kinase NEK2 was identified as a tumor-associated antigen. Direct testing of serum from patients newly diagnosed with neuroblastoma showed specific serological responses in two of 20 patients. Although NEK2 was not universally recognized, it may serve as a tumor antigen for some patients. (*Cancer Sci* 2010; 101: 2316–2324)

**S**erological Screening of Recombinant cDNA Expression Libraries (SEREX) has been a successful strategy for tumor antigen identification, utilizing a prokaryotic expression system and serum from a tumor-bearing host to screen a cDNA library created from that tumor.<sup>(1–3)</sup> SEREX has identified over 2100 tumor antigens, the majority of which are over-expressed non-mutated self-proteins.<sup>(3,4)</sup> Microarray technology, which allows the expression levels of all known transcripts in a tissue sample to be measured, may identify tumor antigens based on this criteria.<sup>(5,6)</sup>

To use expression-profiling data for the discovery of new tumor antigens, we developed a methodology that incorporates both serological immune responses and gene expression analysis, focusing on a small set of tumor-expressed candidate genes, and the immune response to them. Our use of a mammalian gene expression system is more likely to preserve epitopes requiring post-translational modification, shown to be important in antibody recognition of both tumor and viral antigens.<sup>(7–11)</sup>

We used microarray data to identify transcripts that are over-expressed in human neuroblastoma. Mouse homologs of these transcripts were then cloned as a fusion gene with *Renilla* luciferase (Rluc), expressed in a eukaryotic cell line, and then screened for recognition by antibody from tumor-immune mice by luciferase immunoprecipitation (LIPS) assays.<sup>(8,11)</sup> In this manner, tumor-specific transcripts identified by microarray analysis were directly tested for immunogenicity (Fig. 1).

We have previously reported the ability to use a cell-based vaccine to induce immunity to the murine neuroblastoma cell-

line AGN2a by engineering the cell line to express CD80 and CD86 (AGN2a-80/86).<sup>(12)</sup> The vaccine effect is enhanced by depletion of regulatory T cells.<sup>(13)</sup> Vaccinated mice also develop a strong antibody response.<sup>(14)</sup> Here, we identify NEK2 as a target of the vaccine-induced immune response. In some neuroblastoma patients, NEK2-reactive antibody was also detected, confirming our new approach to tumor antigen identification.

## Materials and Methods

**Vaccination and collection of immune mouse serum.** A/J mice (Jackson Labs, Bar Harbor, ME, USA) were housed in the MCW Biomedical Resource Center. All experiments were carried out with approval of the MCW Institutional Animal Care and Use Committee. Mice were separated into four groups: (i) PC61 treatment (purified anti-CD25 monoclonal antibody, 250 µg, injected intraperitoneally on day -10); (ii) vaccination ( $2 \times 10^6$  AGN2a-80/86 cell-line injected subcutaneously on days -7 and 0); (iii) PC61 plus vaccination; and (iv) tumor-bearing ( $10^6$  AGN2a cells injected subcutaneously on day -7). On day 5, blood was collected from the tail vein and serum frozen at  $-80^\circ\text{C}$ .

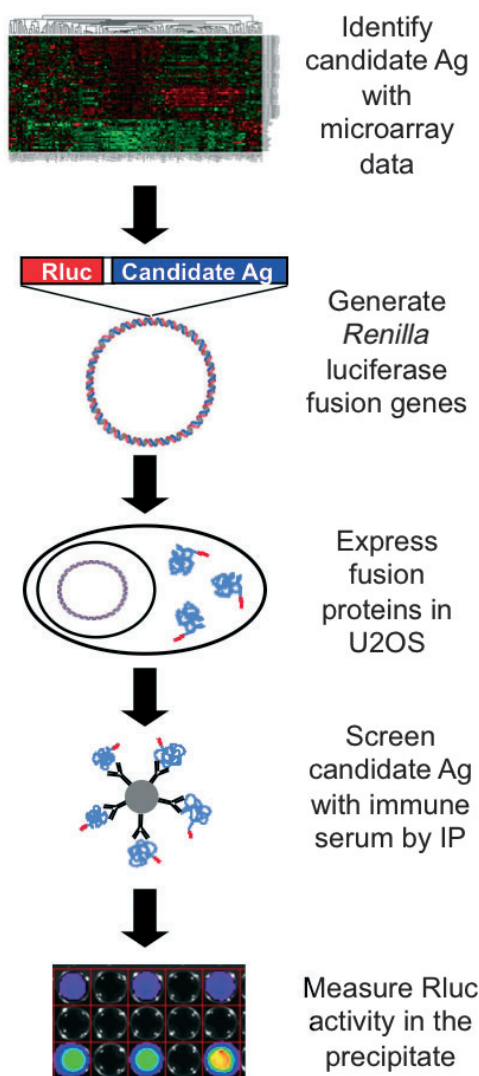
**Cell-lines and cell culture.** The AGN2a cell-line and the AGN2a-CD80/86 vaccine cell-line were cultured in supplemented cDMEM media as previously described.<sup>(12)</sup> The human osteosarcoma cell-line U2OS was cultured in RPMI with 10% FBS (Gemini Bio-Products, West Sacramento, CA, USA), penicillin, streptomycin and *L*-glutamine (Invitrogen, Carlsbad, CA, USA).

**Identification of candidate tumor antigens.** To identify genes over-expressed in neuroblastoma, the Small Round Blue Cell Tumor (SRBCT) database was searched for genes over-expressed in neuroblastoma relative to other tumor types (<http://pob.abcc.ncifcrf.gov/cgi-bin/JK>). The SRBCT-identified neuroblastoma-specific gene list was supplemented with genes previously identified as over-expressed in neuroblastoma.<sup>(15–19)</sup> The complete gene list was then examined in a dataset of gene expression profiles of 43 human neuroblastoma samples and 13 non-neoplastic, control tissue samples using the Affymetrix Genechip human genome U133 AB.<sup>(20)</sup> The .cel files were exported from Affymetrix GCOS software (Santa Clara, CA, USA) and normalized in dChip to the median intensity using the PM-only model.

**RT-PCR and cloning of mouse homologs from the AGN2a cell line.** Mouse homologs of over-expressed transcripts were identified by the GeneCards database and primers designed using e-PCR.<sup>(21,22)</sup> RNA was extracted using Trizol (Invitrogen), DNase treated (Ambion, Austin, TX, USA) and reverse transcribed using Superscript III (Invitrogen). AGN2a cDNA was screened by PCR, with annealing temperatures based on the  $T_m$  of the primer sets. Reactions yielding no product were repeated using varying annealing temperatures ( $45\text{--}70^\circ\text{C}$ ). The forward

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## Bioinformatics-based Ag screening



**Fig. 1.** Bioinformatics-based serological tumor-antigen (Ag) discovery. Gene expression profiling was used to identify over-expressed transcripts in human neuroblastoma. Murine homologs of these genes were cloned to generate fusion genes with *Renilla* luciferase (Rluc). U2OS cells were transfected with the fusion genes and the presence of antibodies against each protein was tested in luciferase immunoprecipitation assays using protein A/G beads coated with serum from mice vaccinated with AGN2a-80/86 cell-line with or without Treg depletion. Precipitation of fusion protein was measured by luminometry, demonstrating the presence/absence of treatment-induced antibody to candidate antigens.

primer for each homolog was designed to insert a *NdeI* or *AseI* restriction enzyme site up stream of the gene. The reverse primers encoded a *BamHI* site, *BglII* site or a *BclI* site downstream of the stop codon. The PCR products were cloned into the TOPO-TA vector (Invitrogen), sequenced, and then used to generate Rluc fusion genes.

**Generation of Rluc candidate antigen fusion genes.** The *Renilla* luciferase (Rluc) gene was PCR amplified from the pHRL-CMV plasmid (Promega, Madison, WI, USA). Primers were designed to add a *NdeI* site and an *XbaI/NheI/SpeI* site upstream of Rluc and to replace the stop codon with a thrombin linker followed by *BamHI* and *NdeI* sites. The PCR products were cloned into TOPO-ZeroBlunt (Invitrogen) and sequenced.

To generate Rluc candidate antigen fusion genes, Rluc was cloned into the *NdeI* site upstream of the candidate antigens (in TOPO-TA). Fusion genes were subcloned into the pcDNA3.1/Hygro(-) mammalian expression vector (Invitrogen) at the *XbaI* and *BamHI* sites. Cloning of tumor-expressed NCAM revealed the presence of three variants. Ncam1(W) encoded the published amino acid sequence, whereas Ncam1(A) and Ncam1(D) each exhibited an insertion at the junction of exons 12 and 13. Ncam1(D) also contained an insertion at the junction of exon 13 and exon 14. Each variant was cloned.

**Expression of Rluc candidate antigen fusion proteins.** U2OS cells were plated in 96-well black plates at  $5 \times 10^4$  cells per well and transfected 24 h later with 0.2  $\mu\text{g}$  pcDNA3.1/Hygro(-) fusion-gene-encoding plasmid and 0.5  $\mu\text{L}$  of Lipofectamine 2000 (Invitrogen). Cells were cultured for 48 h, colenterazine (Biotium, Hayward, CA, USA) was added (1  $\mu\text{g}/\text{mL}$ ), and light production measured on the Lumina *In vivo* Imaging System (Xenogen, Alameda, CA, USA). Light production was observed with each fusion gene.

**Rluc candidate antigen western blot.** Transfected U2OS were harvested, washed twice, and resuspended pellets were boiled in lithium dodecyl sulfate (LDS) sample buffer with a reducing agent (NuPAGE; Invitrogen). Samples were separated on 4–12% Bis-Tris Mini Gels in MOPS SDS running buffer and protein transferred to PVDF membranes (NuPAGE; Invitrogen). Membranes were washed, blocked and probed with a mouse anti-Rluc monoclonal antibody (1:500, 1D5.2; Millipore, Billerica, MA, USA) followed by alkaline phosphatase-labeled rabbit anti-mouse IgG (H + L) antibody (1:100; Abcam, Cambridge, MA, USA). The membrane was washed and incubated in BCIP/NBT substrate (Sigma, St Louis, MO, USA). Blots were stripped with 0.2 M glycine (pH, 2.9) at 55°C, washed and reprobbed with rabbit anti-actin antibody (1:5000; Novus, Littleton, CO, USA) followed by alkaline phosphatase-labeled goat anti-rabbit IgG (1:1000; Santa Cruz Biotech, Santa Cruz, CA, USA) and CDP-Star (Roche, Indianapolis, IN, USA) chemiluminescent reagent.

**Murine luciferase immunoprecipitations.** Five  $\times 10^6$  U2OS cells were plated in a 10  $\text{cm}^2$  tissue culture plate and transfected the following day with 16  $\mu\text{g}$  of fusion gene plasmid and 40  $\mu\text{L}$  Lipofectamine 2000 (Invitrogen), and lysates were collected 48 h later. Protein A/G agarose beads (Calbiochem, Gibbstown, NJ, USA) were washed and loaded with 2  $\mu\text{g}$  of goat anti-mouse IgG (H + L), goat anti-mouse  $\mu$ -chain or goat anti-mouse Fc $\gamma$  heavy chain (Jackson ImmunoResearch, West Grove, PA, USA). Beads were incubated with serum overnight at 4°C while rotating end-over-end. For Bcl-2 dose-response experiments, beads were incubated overnight at 4°C with various amounts of anti-Bcl2 antibody (Abcam) or affinity purified IgG (Jackson ImmunoResearch). Serum-loaded beads were incubated with lysates at 4°C overnight. Beads were pelleted, washed and transferred to a 96-well black plate. Colenterazine was added to each well (1  $\mu\text{g}/\text{mL}$ ) and light production was measured using the Victor3 luminometer (PerkinElmer, Waltham, MA, USA). Readings were recorded in relative light units (RLU) and normalized to no serum controls.

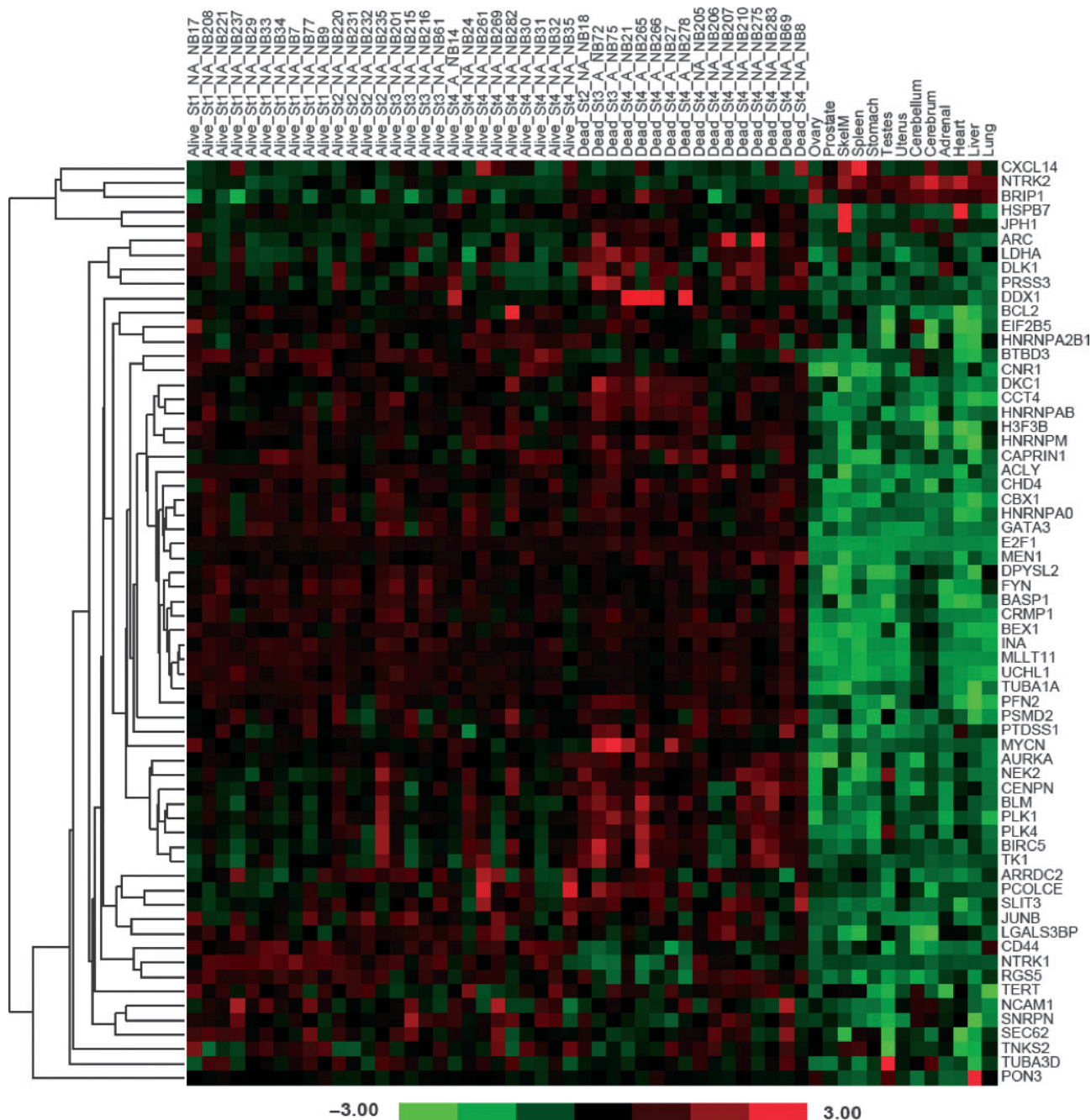
**Human luciferase immunoprecipitations and human serum.** The LIPS assays were performed using protein A/G beads loaded with human serum. The serum was from 20 newly diagnosed neuroblastoma patients, generously provided by the Children's Oncology Group (COG) Neuroblastoma Biology Committee (Dr Michael Hogarty, chair). The control patient serum was generously provided under approved protocol by Dr Paul Sondel, University of Wisconsin School of Medicine (Madison, WI, USA). Immunoprecipitation assays were carried out with lysate from U2OS cells transfected with either NEK2-Rluc (human NEK2 clone NM\_002497.2; Origene Technologies, Rockville, MD, USA) or GFP-Rluc fusion gene plasmids.

**Statistics.** GraphPad Prism (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. For all tests, a *P*-value of <0.05 was considered significant.

## Results

There are few known antigens consistently associated with neuroblastoma, which thus limits the development of antigen-specific immunotherapy.<sup>(23–26)</sup> The SRBCT dataset of the Oncogenomics database was searched for genes over-expressed

in neuroblastoma and 38 potential neuroblastoma antigens were identified. We supplemented this list with 26 genes previously identified as being over-expressed in neuroblastoma, such as survivin and NTRK2.<sup>(16–19)</sup> To determine if these transcripts were over-expressed relative to normal tissues, expression profiling data from clinical neuroblastoma samples were assessed.<sup>(20)</sup> We found that the majority of genes identified as over-expressed in the SRBCT database were also over-expressed in neuroblastoma relative to normal tissues (Figs 2,S1).



**Fig. 2.** Identification of over-expressed genes in neuroblastoma. Gene expression data was mined to identify transcripts over-expressed by neuroblastoma. The gene list was examined in a microarray dataset of 43 clinical neuroblastoma samples and 13 normal tissue samples. For each neuroblastoma sample the clinical outcome (Alive/Dead), clinical stage at the time of collection (St1–St4) and MYCN amplification (A) or non-amplification (NA) was recorded. The heatmap represents the relative expression of these genes in tumor and normal samples. The color map represents the z-scored expression values across all samples.

**Table 1. Murine homologs of human genes over-expressed in neuroblastoma. The AGN2a cell line was screened by RT-PCR for expression of the mouse homologs of the 64 genes identified in Figure 2. Expression (+) or lack thereof (–) was determined by PCR**

Neuroblastoma gene	AGN2a expression
ACLY	+
ARC	+
ARRDC2	–
AURKA	+
BASP1	+
BCL2	+
BEX1	+
BIRC5	+
BLM	+
BRIP1	+
BTBD3	+
CAPRIN1	+
CBX1	–
CCT4	+
CD44	+
CENPN	–
CHD4	+
CNR1	+
CRMP1	+
CXCL14	+
DDX1	+
DKC1	+
DLK1	–
DPYSL2	+
E2F1	+
EIF2B5	+
FYN	+
GATA3	+
H3F3B	+
HNRNPA0	+
HNRNPA2B1	+
HNRNPAB	+
HNRNPM	–
HSPB7	+
INA	+
JPH1	+
JUNB	–
LDHA	+
LGALS3BP	+
MEN1	+
MLLT11	+
MYCN	+
NCAM1	+
NEK2	+
NTRK1	–
NTRK2	+
PCOLCE	+
PFN2	+
PLK1	+
PLK4	+
PON3	–
PRSS3	–
PSMD2	+
PTDSS1	+
RGS5	+
SEC62	+
SLIT3	+
SNRPN	+
TERT	–
TK1	+
TNKS2	–
TUBA1A	+
TUBA3D	+
UCHL1	+

A major aim of the present study was to use a mouse model of tumor immunity to screen potential antigens identified by gene expression profiling in patients. The motivation for using a mouse model of protective immunity was that clinical serum samples are frequently taken prior to treatment. Recently it has been demonstrated that antibodies generated in tumor-bearing hosts recognize antigens to which the immune system had been tolerized.<sup>(27)</sup> The post-vaccination setting in our murine model provides a unique opportunity to identify tumor antigens to which protective responses are mounted. Prior to testing the candidate gene list, we tested AGN2a for expression of the mouse homologs of the genes identified in the clinical samples in Figure 2. AGN2a expressed the majority of these homologs (53/64), establishing them as relevant candidates (Table 1).

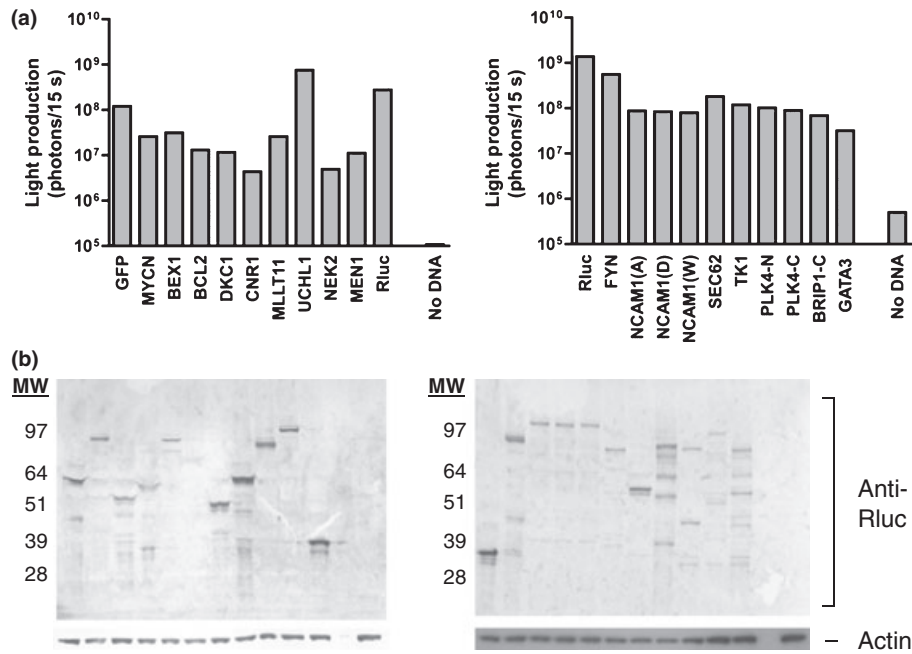
Sixteen of the candidate antigens identified in Figure 2 and Table 1 were PCR amplified and cloned as Rluc fusion genes. Fifteen were cloned in their entirety (Plk4 was cloned as two overlapping fragments, Plk4-N and Plk4-C, encoding the N-terminal and C-terminal halves). For Brip1, 1750 bp of the 3'-region of the coding sequence was cloned, generating Brip1-C, encoding the C-terminal half. Only tumor-derived Ncam1 showed differences in the amino acid sequence from published sequences (as detailed in Methods, each was tested). Fusion genes were transfected into the human U2OS cell-line, colenterazine was added, and light production was measured to test for the presence of fusion proteins (Fig. 3a). The samples were subsequently examined by western blot analysis using an anti-Rluc antibody to verify production of the full-length protein (Fig. 3b).

For standardizing LIPS, protein A/G beads were coated with serial dilutions of an anti-Bcl2 antibody or control IgG and tested with lysate from Rluc-Bcl2-transfected U2OS. Luciferase activity was precipitated by anti-Bcl2 antibody in a dose-dependent manner and was significantly greater than control IgG, giving a detectable signal at a threshold of 1 ng of the precipitating antibody ( $P < 0.05$ , Fig. 4a). To determine if LIPS could be used to identify a model antigen expressed by a cell-based vaccine, we vaccinated mice with GFP-expressing AGN2a-80/86 and screened for a reactive antibody. The GFP-reactive antibody was detected in mice vaccinated with the GFP + AGN2a-80/86 cell-line, but not in the GFP-negative controls (Fig. 4b).

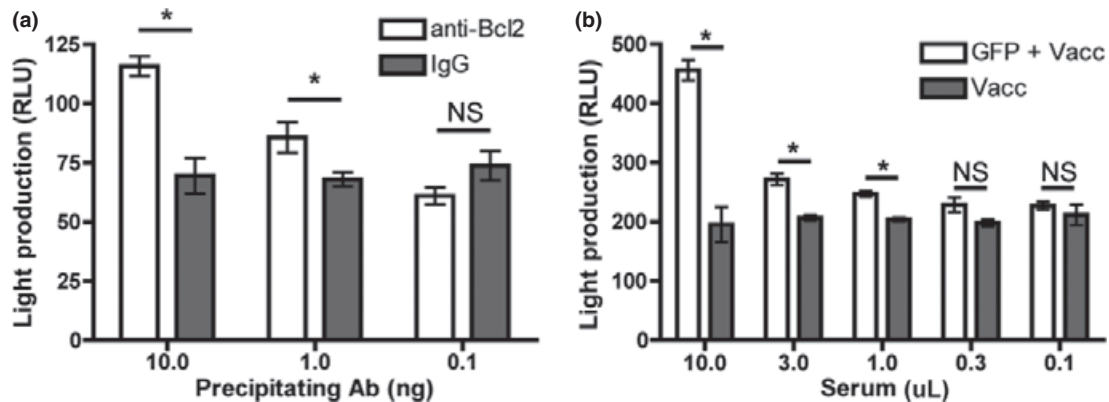
Serum was then collected from mice treated with PC61 and/or vaccination to screen for recognition of the candidate antigens (Fig. 5). Nek2-reactive antibodies were detected in the serum of mice treated with vaccine or with PC61 and vaccine (Fig. 5a). Mice treated with PC61 and vaccine also produced antibodies that precipitated Ncam1 (Fig. 5a).

IgG production is the result of class-switching recombination (CSR).<sup>(28,29)</sup> To examine the production of Nek2- and Ncam1-reactive IgG, we performed LIPS using a secondary antibody specific for mouse IgG. Mice treated with PC61 and vaccine produced significantly more NEK2-reactive IgG than naïve mice or mice treated with PC61 alone ( $P < 0.05$ , Fig. 5b). Mice treated with vaccine alone demonstrated a trend for higher levels of NEK2-reactive IgG relative to naïve or PC61-treated mice (Fig. 5b). Ncam1-reactive IgG was not detected in any of the treatment groups, suggesting that the reactive antibodies were IgM (Fig. 5b).

In mice, pooled serum is commonly used in the initial screening of tumor antigens. While the production of NEK2-reactive IgG was consistently observed in groups of vaccinated mice from multiple experiments, the individual variance of this response was unknown. SEREX and ELISA results from the HER-2/neu breast cancer model suggest that antibody responses to tumor antigens identified with pooled serum may be heterogeneous on a mouse-to-mouse level.<sup>(30)</sup> We found that production of NEK2-reactive IgG was heterogeneous (Fig. 5c). Forty-four percent of mice treated with the vaccine (with or without PC61)



**Fig. 3.** Generation of *Renilla* luciferase (Rluc) candidate antigen fusion proteins. (a) Candidate antigens identified in Figure 2 and Table 1 were cloned in frame with Rluc, U2OS cells were transfected, and Rluc activity at 48 h was tested. Colenterazine was then added and light production was measured. Non-transfected U2OS cells (no DNA) served as a negative control. Results are representative of three experiments. (b) U2OS cells from Figure 3a were lysed, and separated by SDS-PAGE. Protein was transferred to a PVDF membrane and probed with an anti-Rluc antibody. Blots were also probed with an anti-actin antibody as a loading control. Expected molecular weight (MW, kDa): GFP, 58; MYCN, 91; BEX1, 52; BCL2, 58; DKC1, 88; CNR1, 76; MLLT11, 50; UCHL1, 61; NEK2, 85; MEN1, 103; FYN, 82; NCOAM, 105; SEC62, 71; TK1, 54; PLK4-N, 78; PLK4-C, 76; BRIP1-C, 95; GATA3, 88; Rluc, 38.



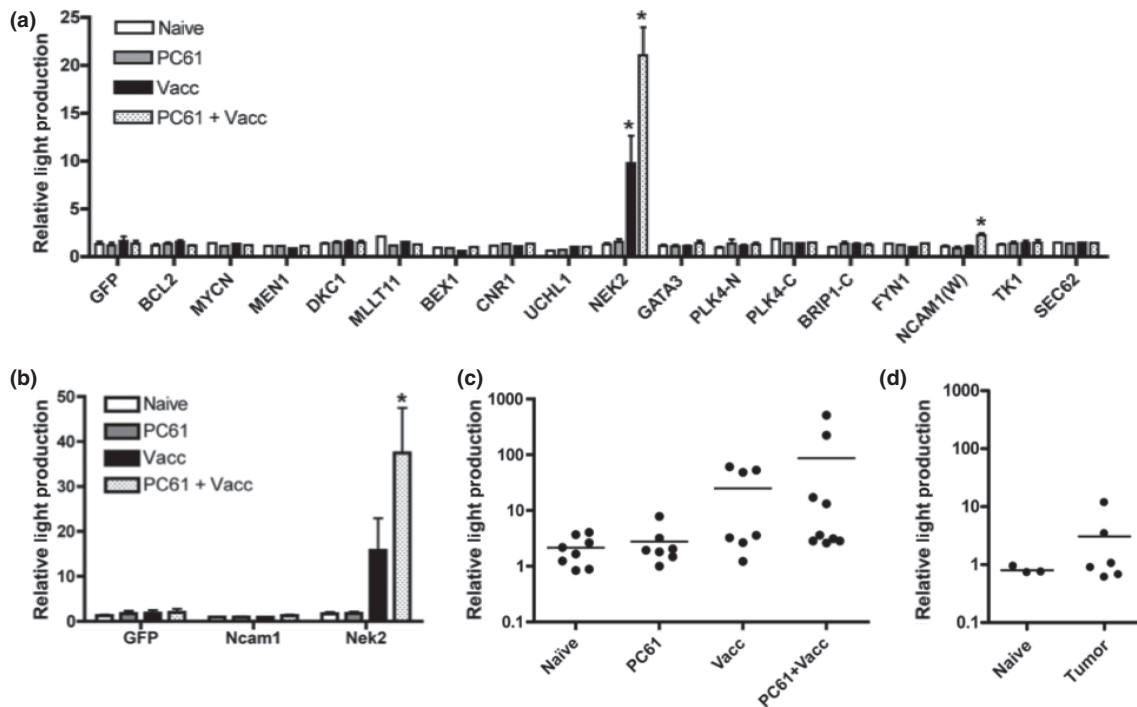
**Fig. 4.** Determining the sensitivity of luciferase immunoprecipitation (LIPS) using anti-Bcl2 antibody and GFP-reactive serum. (a) Protein A/G beads were coated with serial dilutions of anti-Bcl2 antibody or control IgG and used to precipitate *Renilla* luciferase (Rluc)-Bcl2 fusion protein and light production (RLU, relative light units) was measured. (b) Mice were vaccinated with GFP-expressing AGN2a-80/86 cell-line or control AGN2a-80/86 cell-line. Serum was collected, serial dilutions were used to coat protein A/G beads and LIPS was performed. Data were analyzed using an unpaired two-tailed *t*-test. \**P* < 0.05. NS, no significant difference in the means. Error bars represent the SEM.

produced NEK2-reactive IgG at five standard deviations above the mean of naïve mice, demonstrating variability in NEK2 recognition. To determine if the absence of NEK2-reactive IgG was due to a lack of CSR, experiments were repeated to determine the frequency of mice producing NEK2-reactive IgM. Only mice producing NEK2-reactive IgG produced NEK2-reactive IgM (data not shown), suggesting that the variability lies in the initial recognition of NEK2 as an antigen.

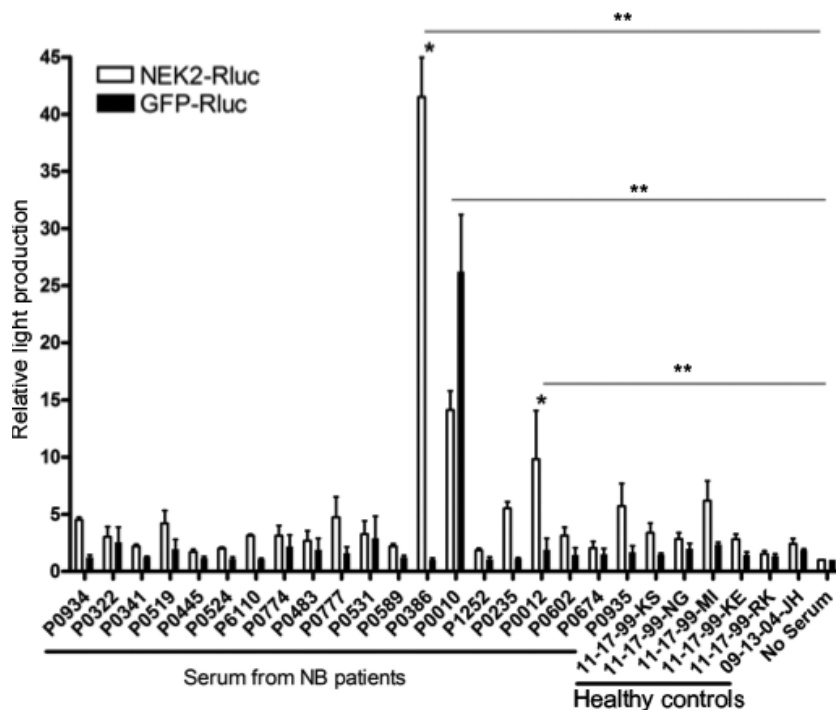
Antibodies against tumor antigens have been observed in cancer patients; however, it is not known if the same antigens are recognized after vaccination with a cell-based vaccine. To test tumor-bearing mice for anti-NEK2 antibody, mice were inoculated with  $10^6$  live AGN2a cells and serum was collected

12 days later. A subset of tumor-bearing mice (33%) produced NEK2-reactive IgG (Fig. 5d). The tumor-bearing mice produced lower levels of NEK2-reactive IgG than vaccinated mice (Fig. 5c,d). Similar to vaccinated mice, only the mice that had generated NEK2-reactive IgG generated NEK2-reactive IgM (data not shown). Thus, immunization with a cell-based vaccine is required for a heightened response.

Antibody responses to tumor antigens is somewhat conserved between species.<sup>(30)</sup> To test our model, human NEK2 (NM\_002497.2) was cloned and tested in the same assay using serum from newly diagnosed neuroblastoma patients (patient details described in Table S1) or from healthy controls. To control for the presence of IgG against the Rluc fragment and/or



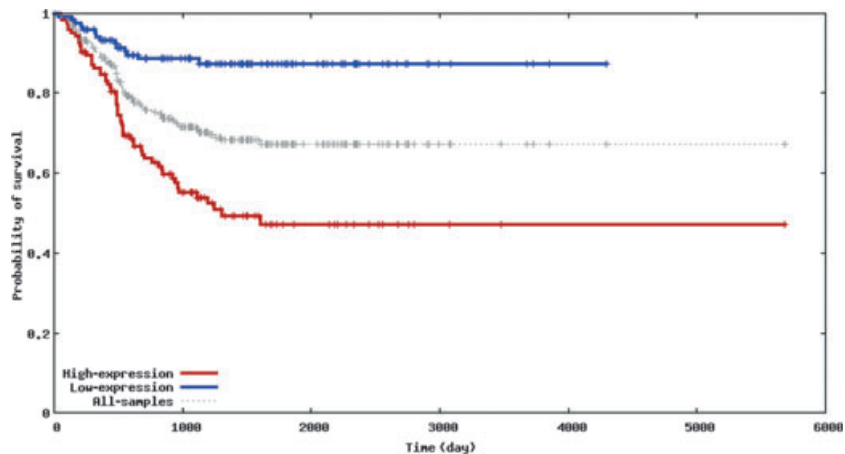
**Fig. 5.** Identification of Nek2 as a tumor antigen. (a) Serum from mice treated with PC61 and/or AGN2a-80/86 cell-line was screened for antibody to the fusion proteins generated in Figure 3. The light production of the precipitated fractions was measured and normalized to the no serum control of each gene. (b) Serum from vaccinated mice was tested for Nek2-reactive IgG. Using an IgG-specific secondary antibody, *Renilla* luciferase (Rluc)-Nek2, Rluc-Ncam1 and Rluc-GFP containing lysates were immunoprecipitated with serum from treated mice. Precipitated fractions were examined for light production and the results were normalized to the no serum controls. Serum from individual treated mice (c) or tumor-bearing mice (d) was then examined for production of Nek2-reactive IgG by luciferase immunoprecipitation (LIPS). Results were analyzed for each gene tested by ANOVA and Dunnett's multiple comparison test. \* $P < 0.05$  relative to naïve. Error bars represent the SEM. Bars in (c) and (d) represent the mean value for the group.



**Fig. 6.** Identification of NEK2-reactive antibodies in the serum of neuroblastoma (NB) patients. Serum from neuroblastoma patients or from healthy (normal) control was used in luciferase immunoprecipitation (LIPS) assays with NEK2-Rluc fusion protein. GFP-Rluc fusion proteins served as a control. Bars represent the median relative light production of three independent experiments. Error bars represent the SEM. \* $P < 0.001$  when comparing serum reactivity against NEK2-Rluc vs GFP-Rluc using two-way ANOVA with Bonferoni post-test. \*\* $P < 0.001$  when comparing serum reactivity against NEK2-Rluc vs no serum control using one-way ANOVA with Bonferoni post-test.

the linker peptide, serum reactivity to GFP-Rluc was also tested. Two of the 20 patient sera demonstrated significant and specific NEK2 reactivity (P0386 and P0235) relative to the no serum

controls (Fig. 6,  $P < 0.001$ ). A third sample (P0010) also recognized GFP-Rluc, suggesting an antibody response to backbone fusion protein elements (Fig. 6). No healthy controls showed



**Fig. 7.** NEK2 over-expression is a marker of poor prognosis in neuroblastoma. The Oberthur Lab Dataset of the Neuroblastoma Prognosis Database (<http://home.ccr.cancer.gov/oncology/oncogenomics/>) was used to compare survival data of patients with NEK2 over-expressing neuroblastoma ( $n = 126$ ) with the survival of patients without NEK2 over-expression ( $n = 125$ ). The Kaplan-Meier plot shows the probability of survival vs survival duration. The difference in survival curves between the two groups was highly significant ( $P = 5.533 \times 10^{-9}$ ) owing to the large number of samples analyzed and large difference in the outcome, demonstrating the depth of this dataset. The generation of this dataset was described previously.<sup>(55)</sup>

NEK2 reactivity. Sample P0386 was found to have significantly more NEK2 reactivity than any of the healthy control sera (Fig. 6,  $P < 0.001$ ). Sample P0235 also demonstrated a significantly higher level of NEK2 reactivity than five of the six healthy control serum samples (Fig. 6,  $P < 0.001$ ).

Finally, we used the Oncogenomics datasets (NCI Pediatric Oncology Branch) to test for the prognostic value of NEK2 expression and to determine if NEK2 expression correlates with an established molecular marker for aggressive neuroblastoma, MYCN amplification. When NEK2 gene expression was divided between high ( $n = 126$ ) and low expressors ( $n = 125$ ), a strong association was seen with high NEK2 expression and decreased survival (Fig. 7). Similarly, in stage 4 MYCN-amplified disease, a strong correlation between NEK2 and MYCN over-expression was seen (Fig. S2).

## Discussion

Tumor antigens include proteins that are mutated, contain unique splice variants, are of viral origin, or that once functioned as differentiation or tissue-restricted antigens.<sup>(31,32)</sup> Genome-scale searches for tumor-specific antigens are being evaluated, but are limited by the difficulty in phrasing search parameters and the small sample size of the primary datasets.<sup>(33)</sup> The over-expression of cancer-associated transcripts can generate cancer antigens by altered glycosylation and expression of unique antigenic epitopes or the inappropriate expression of developmental antigens.<sup>(34-36)</sup>

SEREX is limited by the use of prokaryotic expression systems, and the use of “at diagnosis” serum samples. Tumor-bearing hosts can be tolerant to tumor antigens, and the use of antibodies from these sources may not identify antigens important for tumor rejection.<sup>(27,37-39)</sup> We addressed these issues by developing a novel methodology for the identification of tumor antigens. By using clinical gene expression profiling data, we identified a narrow set of over-expressed genes (Fig. 2). A subset of these genes was then cloned as Rluc fusion proteins (Fig. 3) and screened for antibody recognition in an established murine model of neuroblastoma immunity (Fig. 5a). One potential limitation of our approach is the frequency with which candidate antigens are identified. In our studies, only one of 17 antigens, NEK2, was a strong target (Fig. 5a). While this finding

is significant, a higher throughput approach may prove more effective in detecting new antigens.

The production of NEK2-reactive IgG was observed in mice treated with vaccine and enhanced by the inhibition of regulatory T cells (Fig. 5b). We also examined the production of NEK2-reactive antibodies in mice inoculated with the parental tumor and found the humoral response to NEK2 to be diminished in tumor-bearing mice in comparison with mice vaccinated with AGN2a-CD80/CD86 (Fig. 5c,d). Thus, vaccination and regulatory T-cell inhibition is required for a strong response to this tumor/self antigen.

In the SEREX database hosted by Cancer Immunity (<http://www.cancerimmunity.org>), 11 genes are reported as associated with neuroblastoma. Topoisomerase II $\alpha$  was the most common hit,<sup>(3)</sup> along with three other antigens encoded by the amplified region on 17q, CCX5, HEXIM1 and HOXB6.<sup>(40)</sup> Behrends *et al.*,<sup>(41)</sup> identified 10 antigens by SEREX, including the Hu and NNP-1 neuronal antigens. These studies, and our own published mouse studies, did not detect NEK2.<sup>(42)</sup> However, using gene expression profiling, Landi *et al.*<sup>(43)</sup> found that expression of NEK2 and TTK were key components of a gene expression signature of cigarette smoking, as well as of early stage tumors.

NEK2 plays a regulatory role in cell division.<sup>(44-46)</sup> Over-expression has been reported in several types of cancer and correlates with a poor clinical outcome.<sup>(47-54)</sup> Here, we demonstrate that NEK2 expression is associated with a poor prognosis (Fig. 7 and reference 19), perhaps due to the correlation of NEK2 overexpression with MYCN amplification (Fig. S2). These data suggest that NEK2 is a potential therapeutic target.

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## Disclosure Statement

The authors have no conflict of interest or disclosures.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Candidate gene expression in neuroblastoma tumor tissue and normal human tissue controls. Gene expression profiling data from 43 patients and normal tissue controls (on the top horizontal axis of the figure, patients are listed according to viability; normal tissues are: ovary, prostate, skeletal muscle, spleen, stomach, testes, uterus, cerebellum, cerebrum, adrenal, heart, liver and lung) were assembled from the Oncogenomics Website (<http://pob.abcc.ncifcrf.gov/cgi-bin/JK>) as detailed in Methods. Relative gene expression is presented as a heat-map and hierarchically clustered (left vertical axis), allowing for the identification of a small number of transcripts that are also expressed in normal tissues (bottom right shows the clustered heat-map region for these normal tissues samples).

**Fig. S2.** Correlation of MYCN and NEK2 expression. Using the National Cancer Institute, NIH, USA (NCI) Neuroblastoma cDNA mRNA Expression Database (hosted as described in Table S1), gene expression in normal tissue (a) and stage 4 MYCN-amplified neuroblastoma samples (b) for NEK2 and MYCN was analyzed using the Pearson correlation method. Although trends were seen for other datasets (stage 4 MYCN non-amplified,  $r = 0.277$ ,  $P = 0.063$  and stage 1 non-amplified,  $r = 0.36$ ,  $P = 0.0295$ ), only MYCN amplified stage 4 proved statistically significant.

**Table S1.** Patient data. Serum provided by the Children's Oncology Group (COG) was analyzed for specific patient characteristics. Serum was collected according to original protocol and distributed according to the quality of the submitted sample, and thus not always complete for these specimens. MYCN amplification status, ploidy, INSS stage (International Neuroblastoma Staging System), Shimada histology, and age at diagnosis are provided, as available, for each sample courtesy of Dr. Wendy London, Lead Statistician for Neuroblastoma, Children's Oncology Group.

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